Search result: 1 of 1

(WO/2001/070002) SIGNAL RECORDING OF A RECEPTOR-EFFECTOR-SYSTEM BY AN EXTRACELLULAR PLANAR POTENTIAL-SENSITIVE ELECTRODE

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Applicant: **MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE];** Hofgartenstrasse 8 80539 München (DE).

Inventors: **FROMHERZ, Peter**; Berchemstrasse 97 80686 München (DE).
MEYER, Elisabeth; Gollenbergstrasse 29 82299 Türkenfeld (DE).
STRAUB, Bernhard; Maierhofstrasse 23 81241 München (DE).

Agent: **WEICKMANN Heinrich**; Weickmann & Weickmann Postfach 860 820 81635 München (DE).

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Abstract: The present invention relates to bioelectronic devices comprising living cells which are in operative contact with an extracellular planar potential-sensitive electrode, e.g. a field effect transistor. The cells comprise an ion channel/receptor system which is responsive to stimuli. Thus, the device is suitable as a bioelectronic sensor. The electrode may also have a capacitive stimulating spot, with which the electrical or functional state of the cell or its ion channel/receptor system may be affected.

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Signal recording of a Receptor-Effector-System by an Extracellular Planar Potential-Sensitive Electrode Description The present invention relates to bioelectronic devices comprising living cells which are in operative contact with an extracellular planar potential- sensitive electrode, e. g. a field effect transistor. The cells comprise an ion channel/receptor system which is responsive to stimuli. Thus, the device is suitable as a bioelectronic sensor. The electode may also have a capacitive stimulating spot, with which the electrical or functional state of the cell or its ion channel/receptor system may be affected.

To determine the pharmaceutical effect of test substances, often so-called cellular screening assays are performed in which a cell to be tested containing a receptor system is brought into contact with a test substance in order to examine its function as an effector on the cellular receptor system. These test procedures are often complicated and expensive. Thus, there is a need for devices and methods which allow a quick and efficient screening of many cells. These could simplify the procedure of pharmaceutical tests.

According to the present invention, this problem is solved by combining receptor-effector systems with the functional characteristics of ion channels. The activity of these ion channels is modulated due to the effect of the receptor-effector system. This modulation can be detected by an extracellular planar potential sensitive electrode.

Thus, a subject matter of the present invention is bioelectronic device comprising a) a cell which expresses an ion channel/receptor system wherein said ion channel is responsive to a change in the functional and/or conformational characteristics of the receptor and b) an extracellular planar potential-sensitive electrode wherein the cell is in operative contact with said electrode.

The device of the present invention comprises a living cell. This cell may be a microorganism, e. g. a bacterial cell or a yeast or fungal cell. Preferably, however, the cell is a eukaryotic cell, more preferably, a mammalian cell. Further, it is preferred that the cell overexpresses the ion channel/receptor system, i. e. the cell is manipulated, e. g. by genetic engineering or mutation in a way that components of the ion channel/receptor system are expressed in a higher amount than in a comparative untreated cell. More preferably, the cell is transfected with nucleic acid molecules encoding components of the ion channel/receptor system. In this embodiment of the invention the cell comprises heterologous nucleic molecules which encode at least a part of the components of the ion channel/receptor system and which allow overexpression of said components.

The ion channel/receptor system comprises a polypeptide or a plurality of polypeptides. On the one hand, the ion channel/receptor system comprises an ion channel component, e. g. a polypeptide or a plurality of polypeptides being capable of mediating an ion, i. e. cation and/or anion current through a cell membrane. On the other hand, the ion channel/receptor system comprises a receptor component which is responsive to stimuli. The receptor may be the ion channel or a part of the ion channel. The receptor, however, may be a molecule which is different from the ion channel, which is, however, in operative connection with the ion channel, e. g. a change in the functional and/or conformational state of the receptor results in a change of the functional state of the ion channel thus resulting in a detectable change of ion current through the cell membrane. The stimuli by which the receptor may be mediated are preferably selected from changes in the potential (inside or outside the cell), the presence or absence of effectors, e. g. ligands of the receptor, illumination, mechanical stimulation, stimulation by stimulation spots on the electrode or combinations thereof.

The cell is cultivated on a planar potential-sensitive electrode. Methods of cultivating cells on planar potential-sensitive electrodes are disclosed e. g. in S. Vassanelli, P. Fromherz"Neurons from Rat Brain Coupled to Transistors"Appl. Phys. A 65, 85-88 (1997). By means of these cultivation cells are obtained, which grow on the potential-sensitive regions of the electrode resulting in an operative contact of the cell and the electrode.

The functional characteristics of the ion channels in the cell include an opening of the channels which will cause an ion

current through all participating channels. These ion currents will also flow in the region of operative contact between cell and electrode resulting in a detectable signal which can be measured by the extracellular planar potential-sensitive electrode. The detectable signal may be e. g. a voltage drop due to a junction resistance by the narrow cleft between cell and substrate or the change of the surface potential of the electrode due to diffuse ion concentration changes in the operative contact zone.

A change in functional characteristics e. g. conductivity of the ion channel changes the ion current and therefore the electrical signal detected by the electrode. Since the ion channels are responsive to the effector-receptor system, an alteration in the effector-receptor system will modulate the opening of the ion channels and thus result in a detectable signal.

Ion channels, particularly the gating characteristics thereof, can be modulated by different methods, e. g. by voltage modulation across the membrane (voltage-gated ion channels), by ligands acting on the intracellular and/or extracellular side of the channel (ligand-gated ion channels), by mechanical changes (mechanically-gated ion channels) or by combinations thereof.

Voltage-gated ion channels, i. e. ion channels which are voltage sensitive, will change their conductivity with the potential drop over the membrane - $V_{intra}-V_{extra}$. If the electrolyte, i. e. the culture medium in which the cell is grown, is grounded ($V_{extra} = 0$ mV) this potential drop equals the intracellular membrane ($V_m = V$; "tra). This potential drop may be measured and/or modulated by patch clamp devices, i. e. electrodes which are inserted in or attached to the cell, and allow an adjustment of V_m to a fixed potential. In another embodiment, the conductivity of voltage-gated ion channels may be changed by voltage modulation due to an interaction with other ion channels, e. g. by means of an action potential. V_m is changed due to ion currents flowing into a cell through different ion channels. This co-operation of several ion channels influences the potential drop over the membrane leading in some cases to an action potential. Moreover, the potential difference between intracellular and extracellular side of the membrane may be modulated by using stimulation spots on the electrode.

A stimulation spot may be integrated next to the potential-sensitive electrode being in operative contact to the cell (Stett et al., Phys. Rev. E 55 (1997), 85). Thus, a device with the features of stimulation and recording may be built. A stimulation spot can, e. g. trigger an action potential which then will be recorded by the extracellular electrode.

Ligands can modulate ion channels preferably by two mechanisms, ionotropic and second messenger systems. In an ionotropic system the ligand molecules bind directly to the ion channels and alter their gating characteristics, e. g. intracellular Ca^{2+} shifts the gating curve of some K channels (DiChiara and Reinhard, J. Physio. 489. 2 (1995), 403). In second messenger systems the ligand molecules bind to a receptor which will first trigger some other molecules before the ion channel is influenced, e. g. many glutamate-second messenger systems.

Of course these different methods of modulating ion channels may be combined to create an effective biosensor which may be used for assaying the influence of a change in environmental parameters, e. g. a test substance (effector) on a receptor molecule in the cell.

The coupling of several ion channels may lead to a sudden and specific voltage drop over the membrane of the cell, called an action potential. To release an action potential there are at least two types of channels necessary, e. g. potassium and sodium channels. Both types of channels can be transfected into cells. Alternatively, one may use cell types which already have intrinsic receptors and respond with an action potential, e. g. chromaffin cells or nerve cells. To trigger an action potential one may use one or several of the techniques described above.

The combination of ion channels with the ability to release an action potential and a receptor-effector system is a powerful tool. The advantage of an action potential is the fast and large voltage drop over the membrane which can easily be detected with an extracellular electrode as an"event".

If the signal is very weak, one may use techniques of averaging. Thus, a simple and uncomplicated device and method for assaying substances, if they influence the release of action potentials, is provided.

In a further embodiment of the invention caged probes may be used for a quick release of a large amount of a ligand. Biologically active substances, e. g. Ca^{2+} or the neurotransmitter L-glutamate may be released by UV- illumination, UV-lasers or flashlamps, and act on the receptor.

A specific example of a device according to the present invention are cells which are transfected with the a-and ß-subunits of the voltage-dependent potassium channel hSlo. These cells are cultivated on a field-effect transistor. The characteristic gating curve of the ion channel may be shifted by ß-estradiol (Valverde et al., Science 285 (1999), 1929). This shift corresponds to the opening of ion channels. By changing the extracellular concentration of R-estradiol an ion current will flow and can be detected by the field-effect-transistor under the cells. This system may be used as a sensor for ß-estradiol concentration.

In another preferred embodiment cells are transfected with a nucleic acid encoding an ionotropic receptor for glutamate, e. g. the NMDA receptor.

The ionic flux through the receptor consists of potassium and sodium ions.

This ionic current may be triggered by extracellular glutamate addition and recorded by an extracellular electrode. Thus, this system may be used as a sensor for gluatmate concentration. Some characteristics of NMDA channels show the high suitability to use this channels as a part of a sensor (single channel conductivity 50 pS ; selectivity for cations : K+, Na+ and Ca" ; voltage dependency ; channel will only open in the presence of glycine ; Kandel et al., Neurowissenschaften 236 (1996), Spektrum Verlag).

In an embodiment of the invention a cell which is transfected with receptors for L-glutamate is grown on a chip. A certain amount of L- glutamate is released by flashlight. By this means, the receptor will be opened and an ion current begins to flow. The ion flow may be detected by the electrode. This means that the ion current through the receptor may be triggered by a flashlight.

When a cell is attached to the electrode surface, which may be oxidized silicon, other insulated semiconductors or metal, the cell membrane and the electrode surface are separated by a cleft which may be filled with an electrolyte as illustrated in Figure 1. Thus, a sandwich structure is formed of e. g. silicon, silicon dioxide, cleft, cell membrane and cell interior. The electrode may be integrated on, e. g. embedded in a chip. The chip may comprise further devices such as stimulating spots, transistors etc. Preferably the chip has at least one integrated field-effect transistor comprising at least one source and drain or an electrode as stimulating spot for applying voltages. The potential sensitive electrode, however, may also be a metal electrode which may be integrated on a chip.

The equivalent circuit of the planar core-coat conductor in a cell-silicon junction is shown in Fig. 1a+b. Capacitances are assigned to the membrane and to the oxide in the junction. One or several ion conductances in the attached membrane may be driven by Nernst potentials. The extended cleft is represented by an ohmic conductance.

The free part of the cell is described by a capacitance and one or several ion conductances, too. The ionic and capacitive currents in the circuit determine the intracellular voltage VM and the extracellular voltage Vj in the cleft. The voltage Vj in the junction controls the transistor. It plays the same role as the gate-voltage on the metalized gate of a common MOS- FET.

We apply Kirchhoff's law to the node in the junction of Figure 1b, and obtain Eq. 1 for the voltage Vj, with the membrane capacitance per unit area cM, the ion conductance 91JM unit area area the membrane membrane the junction, the reversal voltage V'o and a cleft conductance gj per unit area of the junction. The approximation of Eq. 1 is valid for weak coupling, i. e. for small values of Vj and dVj/dt at a modest electrode capacitance cOX. dVM gj VJ = g1JM (VM - V01) + CM ------ The properties of the planar core-coat conductor are"squeezed"into the cleft conductance gj per unit area of the junction according to Eq. 2 with the distance d of membrane and substrate, with the specific resistance PJ of the electrolyte in the cleft and with the radius aJ of a circular junction.

5# dj 1 gi, ----- Pi 77a21 We may eliminate the capacitive current in Eq. 1 by taking into account Kirchhoff's law for the intracellular node of Figure 1b. The capacitive current through the total membrane is balanced by the total ion current through the free and the attached areas of the membrane according to Eq.

3, with the specific conductances g'FM and g1JM the two regions regions with the ratio ß of the areas of attached and free membrane. Again the approximation of Eq. 3 is valid for weak coupling, i. e. for small values of Vj and dVj/dt. dVM (1 = - (g1FM + ßg1JM) (VM - V01) dt Inserting Eq. 3 into Eq. 1, taking into account Eq. 2, we obtain the coupling relation for an ionoelectronic sensor according to Eq. 4.

IDJ a2J g1JM g1FM V, ------ ------------- (VM - V01) (4) 5dj 1 + ß The relation shows that a large signal on the gate requires : (i) a small distance d of membrane and substrate, (ii) a large radius aj of the contact, (iii) an enhanced or depleted conductance of the receptor channels in the attached membrane with g1JM -g1FM # 0, and (iv) an electrochemical driving force VM-V10 Thus, the bioelectronic device of the invention is suitable as a sensor which allows the determination of a change in an environmental parameter as a detectable signal on the electrode and which is suitable as a scientific tool for studying the conformational and functional states of membrane proteins.

Particularly, the environmental parameter is an effector for the receptor component of the ion channel/receptor system. More particulary, the system is used to determine, if a test substance is capable of activating or inhibiting the receptor component of the ion channel/receptor system. The receptor component may be a pharmaceutical relevant target molecule.

Thus, the present invention provides a method for contacting a test substance with a bioelectronic device as described above, wherein said bioelectronic device comprises a cell expressing and preferably overexpressing an ion channel/receptor system, wherein a response of the receptor to the test substance is determined by an electric signal in the electrode of the bioelectronic device.

In another embodiment, the bioelectronic device may be used as a sensor to determine the presence or the amount of a substance which acts as an effector to the receptor component of the bioelectronic device.

The method of signal recording as described above can also be used to study the interactions of cells in the situation of contact with extracellular molecules (often mentioned as adhesion region). In biological systems cells have nearly always contact to neighbouring cells or neighbouring medium (for example cell-cell contact between nerve cells in synapses, neuromuscular junctions, gap junctions, cell accumulations of tumor cells, etc.). In all cases the cells are not isolated from each other but have "informational"contact with their environment. Often extracellular molecules transmit this information. For example : the occurance of certain proteins in the synapse of one cells is proposed to be mediated by molecules which are in the plasma membrane of the other cell in contact to the first cell. A depletion, a clustering or an altered functional characteristic of the proteins may be the result of this interaction. Examples of this kind of interactions are described in : trie M., et al. (Science 277, 1511-1515, 1997) and Isom L., et al. (Cell 83, 433-442, 1995).

The system we introduced can study these phenomena by imitating the cell-cell interaction with a cell-substrate interaction. Therefore the substrate e. g. the surface of the chips may be coated by an adhesion molecule of interest, preferably a protein such as collagen IV. The cells which carry the transfected ion channels (or more general : the transfected receptor- effector-system) are cultured on the chip. To open the ion channels and to evaluate the results any method as described above can be used. The properties of the receptor-effector-system with contact to adhesion molecules are obtained. These results can be compared with the results of standard methods (patch clamp, here especially excised patch measurements) to compare the properties of the receptor-effector-system with and without interactions to adhesion molecules.

For example, the surface of a chip was coated with the adhesion molecule col (agen (V and found that the recombinant ion channels were attracted to the surface of adhesion and acculumated there (10 times more ion channels in the region of adhesion than in the free cell membrane).

Further, the system as described above allows an enhancement of the signal/noise ratio of the receptor-effector-system. In principle there are three possible ways to improve the signal/noise ratio : 1) enhancing the intrinsic noise of the used planar electrodes, 2) altering the contact area of the cell/chip interface (reducing the cell- substrate distance or/and enlarging the contact area) and/or 3) clustering the receptor-effector-system of interest in the contact area, wherein the third way is a very powerful method and thus particularly preferred.

A specific clustering of the receptor-effector-system in the region of adhesion (in the cell membrane in contact to the potential sensitive electrode) can enhance the signal/noise ratio. In one example for a collagen IV coated chip it was found that the region of adhesion of the cell membrane there are-1. 4 ion channels/, um2. It is well known that in biological systems the density of receptor-effector-system may be much higher. A famous example are the acetylcholine receptors in the neuromuscular junction, which achieve a density of 10000 receptors/, um2 (Hirokawa and Heuser, J. Neurocytol. 11, 487-510 (1982)). By implementing this or any other mechanism of channel aggregation the signal/noise ratio will be improved by the factor 10000. Ways to achieve this clustering are e. g. the coating of the substrate with specific extracellular adhesion molecules and/or the treatment of the cell with methods of genetic engineering in order to obtain expression of adhesion molecules on the cell surface.

Further, the invention shall be explained by the following figures and examples : Fig. 1 : a) A schematic picture of an ionoelectronic sensor with a cell on an open field-effect transistor. A thin cleft of electrolyte separates the attached membrane from the silicon dioxide of the silicon chip. A chemical signal in the solution opens receptor channels in the free and in the attached membrane. Ionic current flows through the free and attached membrane, driven by a suitable thermodynamic force. The resulting superposition of ionic and capacitive current through the attached membrane flows along the narrow cleft and gives rise there to a voltage drop. The change of extracellular voltage in the cleft plays the role of a gate voltage for the open field-effect transistor and modulates the electronic current from source (S) to drain (D) in the silicon chip. b) Equivalent circuit of the planar core-coat conductor in a cell-transistor junction (point-contact model). The width of the cleft between membrane and chip is blown up. Source (S), drain (D) and bulk silicon (B) are kept at bias voltages with respect to the bath. The intracellular voltage VM and the extracellular voltage Vj depend on the capacities of the attached and free membrane, on one or several ion conductances-driven by Nernst-type batteries-, on the stray capacitance of the chip and on the conductance of the cleft. A substance in the bath which opens ion channels affects conductances in the attached and free membrane. The resulting change of the voltage Vj is detected by the transistor. An injection current IINJ can be applied by an impaled or fused micropipette.

Fig. 2 : A schematic picture of an embodiment of the present invention.

The voltage and calcium dependent human slowpoke (hSlo) potassium channel was expressed in HEK 293 cells. Transfected cells on a gate are contacted with a patch pipette in a whole cell configuration. In the voltage clamp modus the total voltage dependent potassium current of the cell is detected. Simultaneously, the extracellular voltage between cell and gate caused by the local potassium current is determined.

Examples 1. Materials and methods 1. 1 Cells and plasmids HEK 293 cells were cultivated in plastic dishes (Becton Dickinson, Plymouth, UK, No. 110L7807P) in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM, No. 074-02100 A ; Gibco Eggenstein, Germany) with 4. 5 mg/ml glucose, 10% heat-inactivated fetal bovine serum (S181 ; Seromed ; Berlin, Germany), 3. 7 mg/ml $NaHCO_3$ (S5761 Sigma), 2 nM L-gutamine (G2150 ; Sigma), 25 U/ml penicillin and 25 U/ml streptomycin (043-05140 ; Gibco).

The cells were transfected with two plasmids : the hSLo a-subunit construct in the plasmid pcDNA3, which was obtained by cloning the nucleic acids encoding the a-subunit of the human slowpoke potassium channel in the vector pcDNA3. (Invitrogen) (Tseng-Crank J., Foster C. D., Krause J. D., Mertz R., Godinoot N., DiChiara T. J., Reinhart P. H.,"Cloning, Expression and Distribution of Functionally Distinct Ca"Activated K' Channel Isoforms from Human Brain", Neuron, 13, 1315-1330, 1994) and the pEGFP-Cl Plasmid (Clontech, Palo Alto, CA, USA), which contains the GFP gene and serves as a control for successful transfection.

The cells were transfected with the calcium phosphate transfection technique 1 day after replating by coprecipitation with a solution containing both plasmid DNAs.

1. 2 Cell culture on the substrate The transfected cells were cultivated on a collagen-coated silicon chip having 96 field-effect transistors. Green fluorescent cells located on a gate were used for patch-clamp determinations.

2. Results Transfected cells located on a gate were contacted with a patch-clamp in whole-cell configuration. The total voltage dependent potassium current in the cell was determined under voltage-clamp. Further, the change of the extracellular potential caused by the local potassium current between cell and gate was determined. This local potassium current was high enough that transistor signals could be observed without signal averaging.

The specific voltage dependent potassium conductivity in the area of adhesion (area of contact between cell and chip) was obtained by calibrating the extracellular voltage in the junction with the specific cell- chip-contact conductivity and dividing through the distance of voltage and reverse potential. It was found that the functionality of the hSlo potassium channel is not influenced by the adhesion. Thus, a functional bioelectronic device for determining the influence of environmental parameters on an ion channel/receptor system is provided.

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Claims 1. A bioelectronic device comprising (a) a cell which expresses an ion channel/receptor system wherein said ion channel is responsive to a change in the characteristics of the receptor, (b) an extracellular planar potential-sensitive electrode wherein the cell is in operative contact with said electrode.

2. The device of claim 1 wherein said cell is a eukaryotic cell.

3. The device of claim 1 wherein the cell overexpresses said ion channel/receptor system.

4. The device of claim 3 wherein the cell is transfected with nucleic acid molecules encoding components of the ion channnel/receptor system.

5. The device of claim 1 wherein said ion channel is selected from voltage-gated ion channels, ligand-gated ion channels, mechanically-gated ion channels or combinations thereof.

6. The device of claim 1 wherein the ion channel is the potassium channel hSlo.

7. The device of claim 1 wherein the ion channel is the ionotropic glutamate receptor NMDA.

8. The device of claim 1 wherein the potential-sensitive electrode is located on a chip.

9. The device of claim 8 wherein the chip has at least one integrated field-effect transistor comprising at least one source and a drain or an electrode as stimulating spot for applying voltages.

10. The device of claim 9 wherein the cell is in operative contact with at least one gate region between a source and a drain or stimulating spot.

11. The device of claim 1 wherein the potential-sensitive electrode is a metal-electrode.

12. Use of a biolectric device according to any one of claims 1 to 11 as a sensor.

13. The use of claim 12 wherein a change in an environmental parameter is sensed as a detectable signal on the electrode.

14. The use of claim 12 wherein the environmental parameter is an effector for the receptor component of the ion channel/receptor system.

15. The use of claim 14 for the determination if a test substance is capable of activating or inhibiting the receptor component.